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UV-induced Cell Damage is Species-specific among Aquatic Phagotrophic Protists

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ABSTRACT. The sensitivity to ultraviolet radiation (UVR, 280–400 nm) of ten species of freshwater and marine phagotrophic protists was assessed in short-term (4 h) laboratory experiments. Changes in the motility and morphology of the cells, as well as direct quantification of DNA damage, were evaluated. The net amount of cyclobutane pyrimidine dimers formed after exposure of the organisms to a weighted dose (Setlow DNA normalized at 300 nm) of 1.7 kJ m^{-2} was quantified by an immunoassay using a monoclonal specific antibody directed against thymine dimers (T<>Ts). This is the first application of this method to aquatic protists. The results indicated that marine and freshwater heterotrophic nanoflagellates, representatives from the order Kinetoplastida (*Bodo caudatus* and *Bodo saltans*, respectively) accumulate significantly higher DNA damage than protists representatives of the orders Chrysomonadida, Cryptomonadida or Scuticociliatida. The high proportion of A:T bases in the unique kinetoplast DNA, may explain the higher accumulation of T<>Ts found in bodonids. Experiments made with *B. saltans* to study the dynamics of DNA damage accumulation in the presence of UVR and photorepairing light, indicated that the mechanisms of DNA repair in this species are very inefficient. Furthermore, the dramatic changes observed in the cell morphology of *B. saltans* probably compromise its recovery. Our results show that sensitivity to UVR among aquatic phagotrophic protists is species-specific and that different cell targets are affected differently among species. While DNA damage in *B. saltans* was accompanied by motility reduction, altered morphology, and finally mortality, this was not observed in other bodonids as well as in the other species tested.

Key Words. Bodonids, ciliates, CPDs, DNA damage, heterotrophic flagellates, microbial food web, UV-B radiation.

FRESHWATER and marine phagotrophic protists, including heterotrophic nanoflagellates (HNF) and ciliates, are important trophic links within the pelagic food web (Beaver and Crisman 1989; Caron 1991; Porter et al. 1979; Sherr and Sherr 1994). In most aquatic ecosystems, they are the dominant consumers of autotrophic and heterotrophic picoplankton (Caron 1991; Sherr and Sherr 1987). Moreover, aquatic protists through their grazing activity play a crucial role in regulating the abundance, production, cell-size distribution, and taxonomic composition of bacterial assemblages, as well as in nutrient remineralization (Barbeau et al. 1996; Caron 1991; González et al. 1990; Jürgens et al. 1999; Porter et al. 1979; Šimek et al. 1999; Sommaruga and Psenner 1995).

Heterotrophic nanoflagellates and ciliates are ubiquitous in aquatic systems (Finlay et al. 1999; Lee and Patterson 1998; Patterson and Larsen 1991). However, detailed information about species composition for different ecosystems is only partially available (Caron 1997; Foissner and Berger 1996; Laybourn-Parry 1984; Lynn et al. 1991; Pace 1982). Abiotic factors such as temperature, oxygen concentration, suspended solids, salinity, and pH are known to affect the abundance, spatial and temporal distribution, and species composition of free-living protist assemblages (Foissner and Berger 1996; Laybourn-Parry 1984; Laybourn-Parry 1994). Sommaruga et al. (1996) suggested that UV radiation (280–400 nm, UVR) might be another environmental factor influencing the temporal and spatial distribution of aquatic protists. However, information about the effects of UVR on different species or natural assemblages of aquatic phagotrophic protists is scarce (Chatila et al. 1999; Ochs 1997; Ochs and Eddy 1998; Sommaruga et al. 1996; Sommaruga et al. 1999; Wickam and Carsten 1998).

Ultraviolet radiation exerts negative effects on several cell targets like DNA, proteins, pigments, and lipids. Damage to DNA and other cell targets may be caused by direct absorption of UV-B radiation (280–320 nm, UVBR) or indirectly through the generation of reactive oxygen species induced mainly by UV-A radiation (320–400 nm, UVAR; Vincent and Roy 1993). However, the mechanism by which UVR affects an organism will finally depend on the absorption characteristic of the intracellular chromophores, which is directly linked to their chemical structure (Cockell 1998).

Information on UVBR-induced DNA damage is available for several aquatic species including bacteria (Joux et al. 1999), phytoplankton (Buma et al. 1997; Karentz et al. 1991), and zooplankton (Malloy et al. 1997). To our knowledge, however, it has never been estimated directly in aquatic protists. In this study, we addressed the question whether species of aquatic phagotrophic protists exhibit different sensitivity to UVR. Sensitivity was assessed by following the changes in cell morphology and motility, and direct quantification of the DNA damage in short-term experiments with artificial UVR. For the last objective, we estimated the concentration of cyclobutane thymine dimers (T<>Ts) in several protists species using for the first time a method developed in human skin cells. The sensitivity to UVR among protists was species-specific, and the species of kinetoplastid flagellates tested accumulated the highest DNA damage.

MATERIALS AND METHODS

Protists. The following marine (mar) and freshwater (fw) species were tested for their sensitivity to UVR: the heterotrophic flagellates *Paraphysomonas vestita* (mar, Chrysomonadida), *Paraphysomonas imperforata* (mar, Chrysomonadida), *Cafeteria* sp. (mar, Chrysomonadida), *Pseudobodo tremulans* (mar, Chrysomonadida), *Bodo designis* (mar, Kinetoplastida), *Bodo caudatus* (mar, Kinetoplastida), *Bodo saltans* (fw, Kinetoplastida), *Spumella* sp. (fw, Chrysomonadida), *Goniomonas* sp. (fw, Cryptomonadida), and the ciliate *Cyclidium* sp. (fw, Scuticociliatida). All species were tested for changes in motility and morphology. However, measurements of DNA damage were done only for the last five species. The different cultures were kindly supplied by Drs. K. Šimek (Hydrobiological Institute, Czech Republic), D. Caron (Woods Hole Oceanographic Institute, USA), and T. Fenchel (Marine Biological Laboratory, Denmark).

Maintenance of cultures. The cultivation medium was a barley grain infusion consisting of two grains autoclaved in 100 ml of tap water (Chloride: 0.2 mg $liter^{-1}$, conductivity at 25 °C: 220 μS cm^{-1}) for the freshwater species or artificial seawater (HW, Germany) in the case of the marine species, to which the protist cultures plus the indigenous bacteria were transferred. The cultures were grown in a walk-in chamber at 15 ± 1 °C

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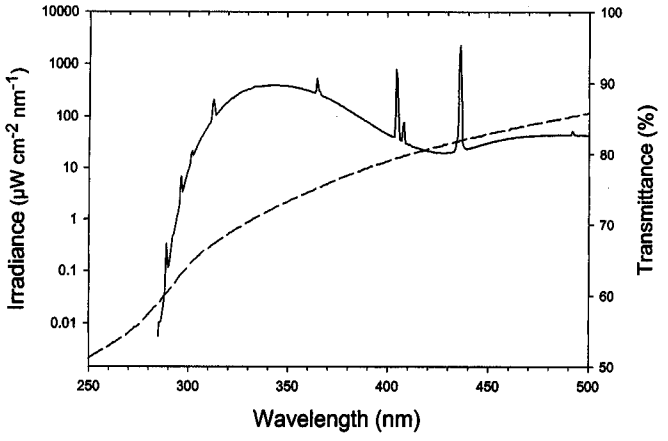

Fig. 1. Spectral irradiance emitted by four Q-Panel A-340 lamps plus two True Lite lamps T12 (continuous line), and transmittance of the medium (dashed line) adjusted to the depth of the medium (2.8 cm). Measurements of irradiance were made at 25-cm distance.

with a light:dark regime of 10:14 h (True Lite T12, 40 W; True Sun, Fairfield, NJ, USA). These lamps emit UV radiation, but mainly in the UVA range. The cultures were maintained for several months under this light regime, before they were used in the experiments. The pH of the medium was 7.5 for the freshwater species and 8.0 for the marine ones. For the experiments, the medium was filtered through a 0.2-μm pore-size filter (polycarbonate, Poretics: Osmonics, Westborough, MA, USA) to remove suspended particles before the inoculation.

Artificial UVR sources. Artificial UV irradiation was provided by a set of 4 tubes of UV-A-340 (Q-Panel Co., Cleveland, OH, USA). These lamps have a maximum emission between 340–345 nm and produce no radiation below 285 nm (Fig. 1). The integration of irradiance values between 280 and 320 nm was 1.69 W m^{-2} (101.4 J m^{-2} min^{-1}). The integral over the same wavelength range for two solar noon spectra measured in June and October at Innsbruck (577 m above sea level, 47° 16′ N) were 2.74 and 0.94 W m^{-2}, respectively (see Sommaruga et al. 1996, Fig. 1). In all experiments, background light for the photorepair process was additionally supplied by two fluorescent tubes (True Lite T12, 40 W; True Sun). The integration of the irradiance between 350–450 nm (i.e. the wavelength range effective for the photoreactivation process, Sancar 1994) was 13.9 W m^{-2}. The UV lamps were preburned for 100 h before the spectrum was measured and the experiments were conducted within the next 50 h. Before the experiment, the lamps were switched on for two hours to reach a constant emission spectrum.

UV measurements. The spectrum of the lamps as well as the solar spectra were measured at high resolution (0.5 nm) with a Bentham DM150 double monochromator spectroradiometer by Dr. Mario Blumthaler (Faculty of Medicine, Institute of Medicine Physics, Innsbruck, Austria).

Experimental design. Experiments were done in the same walk-in chamber where the cultures were grown. Because of the heat produced by the set of lamps, a water bath was used to keep the temperature inside the containers at 16 ± 1 °C. Sterile glass dishes without lids (90-mm diam. and 50-mm height, Schott, Germany) were used to expose the organisms. The distance from the lamps to the liquid surface was 25 cm. At the beginning of the stationary phase, the organisms were exposed (two replicates for each culture) to the full spectrum of the lamps for a maximum of 4 h corresponding to an estimated biological effective dose [Setlow (1974) DNA action spectrum normalized to 300 nm] of 1,690 J m^{-2}. At regular intervals, the liquid in the dishes was gently stirred with a glass stick in order to avoid different exposure inside the container (depth of the liquid: 2.8 cm). The cultures were diluted with sterile tap water or artificial marine water in order to assure a transmittance of 60% at 290 nm at the bottom of the liquid (Fig. 1). After making the dilution, 2–5 ml subsamples were preserved with formaldehyde (2% final concentration) to check the abundance of protists (see below).

Morphology and motility assessment. To test the effect of UVR on the morphology and motility of the protists, portions (200 μl) were collected at 30-min intervals and placed in a double-concavity microscope slide. At each sampling interval, the number of cells showing normal movement (motility) and the morphology of the organisms were recorded (Sommaruga et al. 1996). Organisms kept in the darkness were used as a control. The protists were observed with a Zeiss Axiovert 135 inverted microscope and their movement registered with a Sony CCD/RGB camera connected to a video system. At least 50 cells were observed at each sampling interval in each treatment.

DNA damage experiment. After the 4-h UV exposure, the whole volume of each container (200 ml) was filtered using low pressure (0.2 atm) through a Whatman GF/C filter (47 mm diam.) in the case of the heterotrophic flagellates or through a 3-μm-pore-size filter (Poretics, 47-mm diam.) for ciliates. The efficiency of the filters to retain the protists was previously checked by filtering 30 ml of the filtrate onto a 0.2-μm-pore-size black Poretics filter and inspecting the filter in an epifluorescence microscope (see below). The filtration step was done in the darkness and care was taken to avoid direct light exposure of the filters during manipulation. To determine the background level of DNA damage in each species, the cultures were kept in the darkness for 4 h and afterwards they were filtered as above. The samples were immediately preserved in liquid nitrogen. DNA damage was quantified within three weeks.

Because the Whatman GF/C filters will retain a certain number of bacteria, we tested the importance of their contribution to the estimation of DNA damage. For this purpose, bacteria from the different cultures were grown under the same conditions as described above but in the absence of predators. The bacteria were exposed under the lamps or kept in the dark for the same period as above.

Quantification of cyclobutane thymine dimers (T<>Ts). The type of DNA damage observed depends on the wavelengths and the dose received. While UVAR induces the formation of DNA strand breaks, DNA protein crosslinks, and alkali-labile sites, UVBR is very efficient in causing dimerization between adjacent pyrimidine bases (Mitchell and Karentz 1993). Two photoproducts are known to be induced by UVBR, the cyclobutyl pyrimidine dimers (CPDs, mainly TT or CT dimers) and the pyrimidine (6–4) pyrimidone (Mitchell and Karentz 1993). However, the quantum yield (photoproducts formed per photon absorbed) at 254 nm is ~ 17 times higher for the CPDs than for (6–4) products (Görner 1994). Consequently, CPDs make up ~ 70–90% of the total UVBR-induced damage photoproducts (Mitchell and Nairn 1989).

The protocol used to determine the concentration of T<>Ts in DNA from phagotrophic protists is a modification of the method described by Vink et al. (1994). DNA was extracted using a modified method from Doyle and Doyle (1987). Filters were incubated at 60 °C for 30 min with 750 μl preheated CTAB isolation buffer [2% (w/v) CTAB (Sigma Chem. Co., St. Louis, MO), 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0]. Then, 750 μl CIA [chloroform/isoamylalcohol (24:1)] was added to extract the DNA from cell debris and proteins. After centrifugation (20,000

g, 10 min), 0.5 ml (2/3 of total vol.) of cold isopropanol was added to the upper (water) phase to precipitate the DNA (1 h, 4 °C). After centrifugation (20,000 *g*, 30 min, at 4 °C), the supernatant was removed and the pellet was washed with 1 ml of 80% ice-cold ethanol (15 min, −20 °C, followed by centrifugation, 30 min, 4 °C). Finally, the DNA pellet was dried under vacuum and resuspended in TE buffer (1mM Tris-HCl pH 8.0, 0.1 mM EDTA). To remove RNA, the DNA was incubated for 1 h at room temperature with 75 μg ml^{-1} RNAse (Boehringer, Mannheim, Germany).

The concentration of DNA was determined fluorometrically using Picogreen dsDNA quantitation reagent (dilution 1:400, Molecular Probes, Leiden, The Netherlands) on a 1420 Victor multilabel counter (excitation 485 nm, emission 535 nm, EG&G Wallac, Dassel, Germany). The concentration of cyclobutane pyrimidine dimers (CPDs) in DNA was determined using an immunodotblot procedure (Boelen et al. 1999) with minor modifications. Briefly, heat-denaturated DNA samples (100 ng) were blotted in duplicate onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany, 0.1 μm). To detect CPDs, the membrane was incubated with the primary antibody H3 (Roza et al. 1988). This was followed by incubation with the secondary antibody (rabbit anti-mouse) coupled to horseradish peroxidase. Chemiluminiscent detection was done using ECL detection reagents (Amersham, Freiburg, Germany, RPN2106) in combination with a photographic film. The films were developed, scanned (UMAX PS-2400X scanner), and analyzed with Image Quant (version 4.2) software from Molecular Dynamics (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The H3 antibody is raised against cyclobutane thymine dimers, but also has a high affinity for 5'TC dimers (Fekete et al. 1998). Since H3 does not bind to all possible CPDs (including CC and CT) we have chosen to refer to T<>Ts instead of CPDs in the following sections.

To allow quantification of the amount of damage in the sample DNA, the samples were compared with a dilution series of standard DNA, which was blotted on the same film. The amount of CPDs in the standard DNA was determined before by calibrating it against DNA isolated from irradiated Hela cells (kindly provided by Dr. Len Roza). The amount of CPDs in this DNA was determined by Roza et al. (1988) by means of HPLC. The detection limit of the CPD assay is < 0.5 CPD/10^6 nucleotides.

Boelen et al. (1999) showed that there is a strong positive linear relationship between the UV dose applied to the DNA and the gray value measured in the same photographic film used in the present study (their Fig. 2). The results were expressed as the concentration of T<>Ts per 10^6 nucleotides (mean of two measurements).

Net DNA damage accumulation in *B. saltans*. To study the dynamics of the net accumulation of T<>Ts in this species, a time series experiment was included. The experiment was done as described above but samples were filtered at time 0, 1 h, 2 h, and 4 h. The background level of DNA damage in this experiment was determined as described above after 4 h. The transmittance of the medium used in this experiment was 76% at 290 nm.

Enumeration of protists. The growth of the different species in the barley grain infusion was monitored by making direct counts under an epifluorescent microscope. Subsamples of 2–5 ml were fixed with formaldehyde (2% final concentration), filtered onto 1-μm pore-size black Poretics filters, and stained with 4',6-diamidino-2-phenylindole (DAPI) according to the method of Porter and Feig (1980). DAPI-stained protists were counted at 1,250× magnification in a Zeiss epifluorescence microscope with a BP 365 excitation filter, FT 395 chromatic beam splitter, and LP 397 barrier filter.

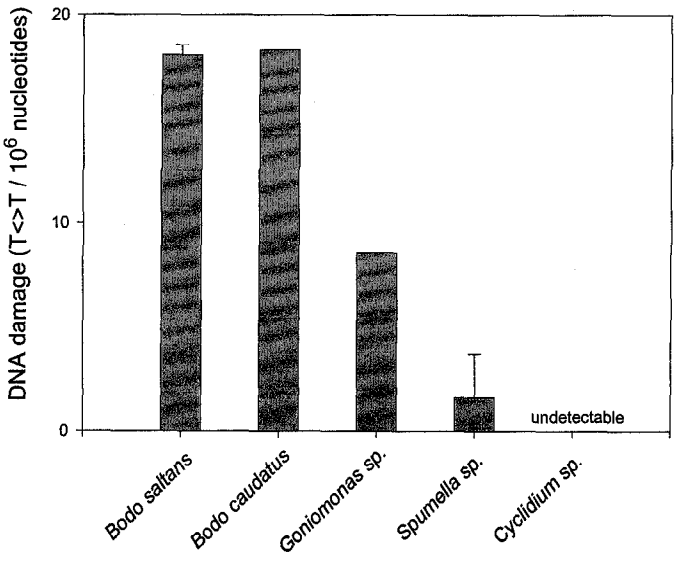

Fig. 2. Net accumulation of cyclobutane thymine dimers (T<>T) in five species of aquatic protists after 4-h exposure to artificial UV radiation equivalent to a biologically effective dose of 1,690 J m^{-2} (Setlow DNA action spectrum normalized at 300 nm). Mean of two replicates ± one standard deviation. When error bars are not shown, the standard deviation was too small to be illustrated.

Statistical analysis. Differences in DNA damage among species were tested using Kruskal-Wallis one-way ANOVA on ranks followed by post-hoc comparisons with Student-Newman-Keuls (SNK) test (Zar 1984). Differences in the median number of motile cells between the UV-exposed protozoa and those kept in the dark were tested with a Mann-Whitney *U* test.

RESULTS

Effects on the morphology and motility of protists. The only species that presented both an altered morphology and motility after UV exposure was *B. saltans*. All the other species showed no sign of UV effect on the morphology or there was no significant difference ($P > 0.05$, Mann Whitney *U* test) in the median number of motile cells when compared with the dark control. The only exception was *Goniomonas* sp., which after 4-h exposure, presented a slight reduction in motility (i.e. 25% of the organisms did not move, data not shown).

Controls and background levels of DNA damage. The filters used to collect the HNF and the ciliate species retained the organisms with 100% efficiency. Background levels of DNA damage in the cultures (i.e. organisms kept always in the dark) ranged from undetectable values to a maximum of 2.0 T<>Ts per 10^6 nucleotides (*Goniomonas* sp.). In this case, the background value was subtracted from the value of DNA damage obtained after UV exposure. The controls performed to check for the possibility of error in the measurements of DNA damage introduced by bacteria retained on the filter indicated a negligible contribution (< 0.7 T<>Ts per 10^6 nucleotides). Nevertheless, these values were also subtracted from the total DNA damage concentration.

DNA damage in protists. The highest net accumulation of T<>Ts after 4-h exposure was found in the two kinetoplastid flagellates, the freshwater *B. saltans* and the marine *B. caudatus* (18.1 and 18.3 T<>Ts per 10^6 nucleotides, respectively). In decreasing order of sensitivity, the flagellates *Goniomonas* sp. and *Spumella* sp. accumulated over the same period of expo-

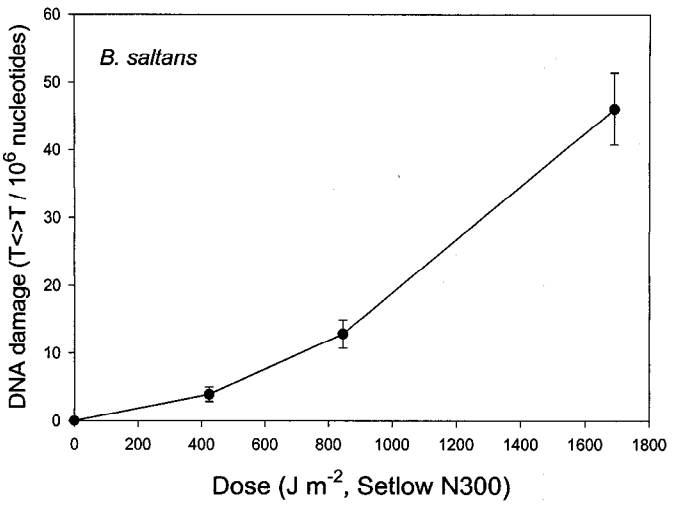

Fig. 3. Dynamics of DNA damage accumulation versus biologically effective dose in the kinetoplastid heterotrophic nanoflagellate *Bodo saltans*.

sure, 52% and 91% lower DNA damage, respectively, than the kinetoplastid flagellates (Fig. 2). T<>Ts were not detected in the ciliate *Cyclidium* sp. The net accumulation of T<>Ts was highly significantly different (ANOVA on ranks, $P < 0.001$) among the species. Post-hoc comparisons showed no significant differences ($P > 0.05$) between the two kinetoplastid species, but there were significant differences among all possible combinations of the rest.

Net DNA damage accumulation in *B. saltans*. The results from this experiment showed that net accumulation of T<>Ts in the DNA of this species increased as a power function of the biological effective dose received (Fig. 3). Only during the first hour of exposure, the relationship between these variables was linear. The higher UVR transmittance of the medium used in this experiment (e.g. 76% at 290 nm) resulted in 2.5 times more DNA damage accumulated after 4 h than in previous experiments with the same species (see Fig. 2).

DISCUSSION

UV effect on the morphology and motility of protists. Changes in swimming speed, motility, and morphology are important for the ability of phagotrophic protists to localize and ingest their prey. A previous study of the effect of UVR on the kinetoplastid HNF *B. saltans* (Sommaruga et al. 1996) showed that UVBR reduces first its swimming speed, then the number of motile cells, and finally causes a dramatic change in cell morphology (i.e. the typical elongated shape changed to a spherical form). Furthermore, this change in morphology is also observed, although to a lower extent, when the organisms are exposed to solar UVAR plus photosynthetically active radiation. These spherical cells are not able to feed upon bacteria and do not recover even after an extended period under photorepairing light (Sommaruga et al. 1996). Our results confirm the sensitivity of this species. However, they also demonstrated that it is not widespread among other members of the same family: the motility and morphology of the marine HNFs *B. designis* and *B. caudatus* were not affected by UVR. At present, there is no clear explanation for the different sensitivity found among these marine and freshwater species of bodonids. The other species of HNF tested and the ciliate *Cyclidium* sp. did not show negative effects regarding these parameters. The only exception was the HNF *Goniomonas* sp., which presented a noticeable reduction in the number of motile cells, but only after 4-h exposure to UVR. Our results confirm previous studies on the marine HNF *P. imperforata* that reported no significant effects of UVR on its motility (Ochs 1997).

DNA damage in aquatic protists. The method adapted in the present study to quantify DNA damage in protists proved to be robust and sensitive as indicated by the low variability between replicates and the low concentration of T<>Ts detected. The levels of DNA damage found in our study (Fig. 2) are within the range of those values measured with the same antibody approach in naked DNA exposed at different depths in the sea (Boelen et al. 1999), indicating that the biological effective dose applied in our experiments was realistic.

Because the organisms were exposed concomitantly to UVR and photoreactivating wavelengths, the accumulation of T<>Ts is the net result of the balance between DNA damage and photorepair, including other mechanisms of recovery like nucleotide excision-repair (dark repair). The results from the DNA damage experiments clearly indicated the existence of significant differences in UVR sensitivity among the species tested (Fig. 2). Species-specific differences in DNA damage and repair have been described for other groups of planktonic organisms like bacteria (Joux et al. 1999), diatoms (Karentz et al. 1991), and zooplankton (Malloy et al. 1997; Zagarese et al. 1997). The two species of kinetoplastid heterotrophic flagellates tested, *B. saltans* and *B. caudatus* accumulated a significantly higher concentration of T<>Ts than the other species when exposed at the same UVBR dose and in a medium with the same UV transmittance. The method used by us to quantify DNA damage cannot identify the contribution of the nuclear DNA and kinetoplast DNA (only present in species of the order Kinetoplatida) to the total concentration of T<>Ts. However, one possible explanation for the higher accumulation of T<>Ts in bodonids is that kinetoplast DNA is A-T rich (Vickerman 1991). A high A-T content is known to make DNA prone to UVBR damage because it increases the possibility of thymine dimerization (Setlow 1966). Another non-exclusive explanation for the high accumulation of DNA damage in these species is the lack of an efficient repair mechanism. Although our experiment was not designed to test the efficiency of the different repair mechanisms, the results on the dynamics of DNA damage accumulation in *B. saltans* suggest an inefficient mechanism of repair in this species. We suggest that the inefficient recovery of this species is also a consequence of the damage caused by UVR to other important cell targets like membranes that dramatically affect the structural stability of the cell.

Our cultures were grown for several months under a dark: light regime including wavelengths in the UVA range. However, they were not exposed to high UVB intensities until the experiments. Lack of acclimation may have exacerbated the negative effects of UVB radiation observed in our study. However, mechanisms of acclimation to high UVR are known for phototrophic flagellates (e.g. production of intracellular UV-absorbing compounds know as mycosporine-like amino acids or MAAs, Carreto et al. 1990) but not for heterotrophic protists. In fact, analyses made to test for the presence of MAAs in the HNF and ciliate species used in our study gave negative results (data not shown).

The organisms tested in our study represent only a small sample of the large diversity of protists species found in freshwater and marine ecosystems. Some of these species are described as ubiquitous, like the marine flagellate *P. tremulans* (Fenchel 1986) or the freshwater flagellate *Spumella* sp. (Laybourn-Parry 1994). Others, like the marine flagellate *P. imperforata* are considered opportunist or 'weeds' (Lim et al.1997), while *B. saltans* is typically found in wastewaters (Laybourn-

Parry 1984) or associated with aggregates (Mitchell et al. 1988). Although protist sensitivity to UVR may be related to their temporal and spatial distribution, as well as to the UV attenuation characteristics of the aquatic systems where they live, our results support the idea that UV radiation was probably an important selective agent during protistan evolution (Rothschild 1999).

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